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Two sites in the third inner loop of the dopamine D_2 receptor are involved in functional G protein-mediated coupling to adenylate cyclase

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Synthetic peptides, corresponding to the amino acid sequences of the N- and C-terminal parts of the 3rd intracellular loop of the dopamine D_2 receptor, attenuate dopaminergic adenylate cyclase inhibition in membranes. Both peptides also activate directly GTPase activity in membranes. We suggest a functional model for G_i-coupled receptors where two sites in the 3rd inner loop compose the links for the receptor–G protein interaction thus providing the tools for a selective and adjustable response. Functional coupling was not affected by a peptide representing the insert in the long form of the dopamine D_2 receptor ($D_{2(long)}$). The selectivity pattern of conventional G protein-linked receptors also sheds some light on the recently observed interaction of β-amyloid protein precursor (APP) complexes with G proteins.

Dopamine D_2 receptor; G protein; Receptor–G protein coupling; Synthetic peptide

1. INTRODUCTION

Transmembrane signaling of G protein-linked receptors proceeds by receptor–G protein–effector assemblies coupled through specific protein–protein interactions along the inner surface of membranes. Heterotrimeric regulatory G proteins not only act as proportionate transducers of hormone signals, but also allow for additional flexibility in adjusting the intracellular response of a variety of effectors to a variety of external signals. Present evidence suggests that the specificity and selectivity of receptor–G protein interactions are determined by the topology, number and composition of potential interaction sites. On the assumption that synthetic peptides making up all or part of the presumptive receptor–G protein binding sites retain structural and functional properties of their origin and thus mimic or compete with the receptor, we have previously analyzed the inner loop regions of β-adrenergic receptors (βAR) for potential interaction sites in signal transmission involving the stimulatory G protein, G_s [1–3]. G protein associated functions were either decoupled or activated. Disclosing interaction sites with site-specific synthetic peptides proved to be useful and versatile also with other G protein-linked receptors [4–6] and for cryptic sequences in G protein-activating proteins like growth factor receptors [7] and the β-amyloid precursor protein (APP) [8].

In contrast to hormonal stimulation of adenylate cyclase, the components and the sequence of interactions in adenylate cyclase inhibition are poorly understood or as yet undetermined [9,10]. In search for the G protein interaction sites of inhibitory receptors we investigated dopaminergic adenylate cyclase inhibition in intact membranes.

The dopamine D_2 receptor (D_2R), which is a subtype of dopamine receptors [11] attenuates adenylate cyclase activity and modulates other effects. The sequence and structure of the D_2R have been deduced from the corresponding cDNA [12, for review see 13]. In common with the topography of the G protein-linked receptor family, seven transmembrane domains connected by intra- and extracellular loops have been suggested [14,15]. The D_2R exists in two forms, $D_{2(long)}$ and $D_{2(short)}$, which are generated from the same gene by alternative splicing, and which differ from each other only by a 29 amino acid insertion in the third intracellular loop [13,16]. This insertion is thought to alter the coupling efficiency of the two receptor subtypes with G proteins [16,17]. G_{i2} has been proposed as a candidate in D_2R–G protein-linked signal transduction [18].

In the present study we were able to identify two G protein interacting regions in the third inner loop of D_2R by specific effects on downstream effectors.

2. MATERIALS AND METHODS

2.1. *Materials*

Peptide synthesis chemicals were from Applied Biosystems and Novabiochem. Radiochemicals were from New England Nuclear.

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Abbreviations: G protein, regulatory guanine nucleotide-binding protein; G_s, stimulatory G protein; $G_{i,o}$, pertussis toxin sensitive G proteins; G_t, transducin; αAR, βAR, α-, β-adrenergic receptor; D_2R, dopamine D_2 receptor; MAChR, muscarinic acetylcholin receptor; PGE_1, prostaglandin E_1.

Human embryonic kidney 293 cells transfected with and stably expressing the $D_{2(long)}$ or $D_{2(short)}$ subtype of the D_2R were obtained from Dr. P. Seeburg [16] and cultured by Dr. T. Voss (Bender & Co. Wien).

2.2. *Peptide synthesis*

Peptides were synthesized by the solid-phase Merrifield method on an Applied Biosystems 430A peptide synthesizer as described earlier [3]. Purity was based on analytical HPLC, and verified by amino acid analysis (W. Hoppe, Universität Würzburg) and FAB mass spectrometry (K.D. Klöppel, GBF, Braunschweig).

2.3. *Membrane preparation*

Membranes were prepared from monolayer cultures of 293 cells. Cells were resuspended with a Polytron homogenizer at setting 6 for 20 s in phosphate-buffered saline (pH 7.4).

2.4. *Ligand binding*

Membranes (50 μg per assay) were incubated for 15 min at 37°C in 50 mM Tris (pH 7.7) with 0.5 nM [^{3}H]spiperone with or without 10 μM haloperidol to calculate the amount of specific binding. The total concentration of receptor-specific binding sites was 3 ± 0.5 pmol/mg membrane protein. Non-transfected cells did not show any specific [^{3}H]spiperone binding.

2.5. *Adenylate cyclase activity*

Adenylate cyclase activity was assayed in membrane suspensions (50–70 μg protein/assay) at 30°C for 20 min as described in [3]. Hormone-induced activation of the adenylate cyclase system was primed with 1 μM PGE_1 and 50 μM GTP with or without 100 μM dopamine. Synthetic peptides were first incubated with membranes for 60 min at 4°C. For controls, a D_2-selective antagonist, 10 μM haloperidol or 10 μM (+/−)sulpiride was added.

2.6. *GTPase assay*

GTPase activity was determined essentially as described in [19] and [20]. Synthetic peptides were first incubated with membranes for 60 min at 4°C. GTPase was stimulated by addition of 100 μM dopamine in the presence of 10 μM propranolol; basal GTPase activity was measured in the presence of 10 μM haloperidol and 10 μM propranolol. The dopamine-stimulated GTPase activity was usually 2-fold larger than the basal GTPase activity and was set to 100%.

3. RESULTS

Based on our previous studies with synthetic peptides on receptor–G protein interactions [3] we made a search for conserved structural motifs [21] within the suggested structure of the D_2R (Fig. 1) by sequence alignment to the corresponding regions in βAR. The peptides selected for synthesis (see below) cover the complete intracellular loop i2 (i2), the N-terminal part of loop i3 (i3N), one half of the unique 'insertion' of 29 amino acids in the loop i3 of the $D_{2(long)}$ receptor (i3I), the C-terminal part of loop i3 (i3C) and the COOH-terminus distal to the seventh transmembrane region, forming a putative loop (i4). In addition, all peptides except D2/256–270 retain 1–5 amino acids of the predicted transmembrane α-helical regions of the receptor [15,16].

D2/128–152 (i2): ISIDRYTAVAMPMLYNTRYSSKRRV;

D2/208–226 (i3N): VYIKIYIVLRRRRKRVNTK;

D2/256–270 (i3I): IMKSNGSFPVNRRRV;

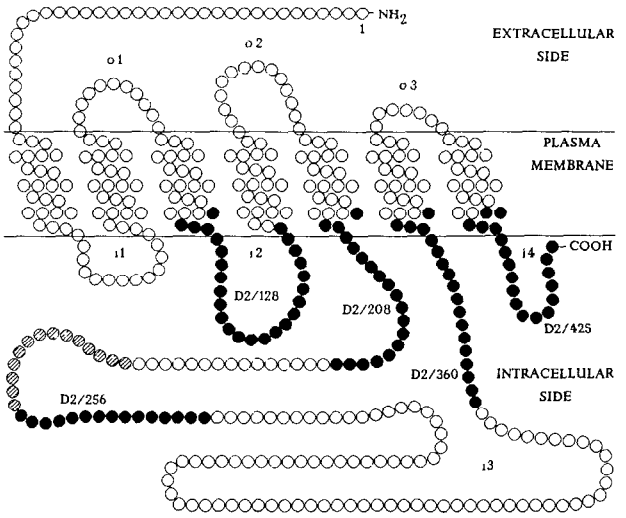

Fig. 1. Location of synthetic peptides with respect to the transmembrane topology of the human dopamine D_2 receptor [15]. The first amino acid of the fragment is indexed, the complete sequences are shown as solid circles. Amino acids representing the 'insert' in the $D_{2(long)}$ isoform of D_2R are marked by shaded and solid circles.

D2/360–377 (i3C): RRKLSQQKEKKATQMLAI;

D2/425–443 (i4): IYTTFNIEFRKAFLKILHC;

3.1. *D2-peptides response studies and controls*

The effects of peptides were studied in membranes of 293 cells, which were transfected with and stably expressed the longer or the shorter subtype of the D_2R at similar levels (~ 10^5/cell); membranes from non-transfected cells served as a control. We found that within the experimental limits peptide effects were the same whatever the receptor subtype, therefore only results from membranes using cells transfected with $D_{2(long)}$ receptors are presented. With respect to possible interference by interactions with related G protein-coupled receptor complexes, controls were set up in membranes from non-transfected cells (Fig. 2A). In the absence of dopamine and at low concentrations all D2-peptides except for D2/208–226 do not interfere with prestimulated adenylate cyclase. The latter peptide is able to stimulate or inhibit adenylate cyclase activities even in non-transfected 293 cell membranes. Above 10 μM concentrations all peptides apparently inhibit adenylate cyclase in prestimulated membranes.

3.2. *Effects of D2-peptides on G protein mediated adenylate cyclase inhibition*

The competence of peptides to uncouple the inhibitory effect of dopamine was judged from their potential to neutralize the dopamine dependent inhibition with the results shown in Fig. 2B–D. In membranes from transfected 293 cells dose-dependent effects were observed at low peptide concentrations with D2/208–226

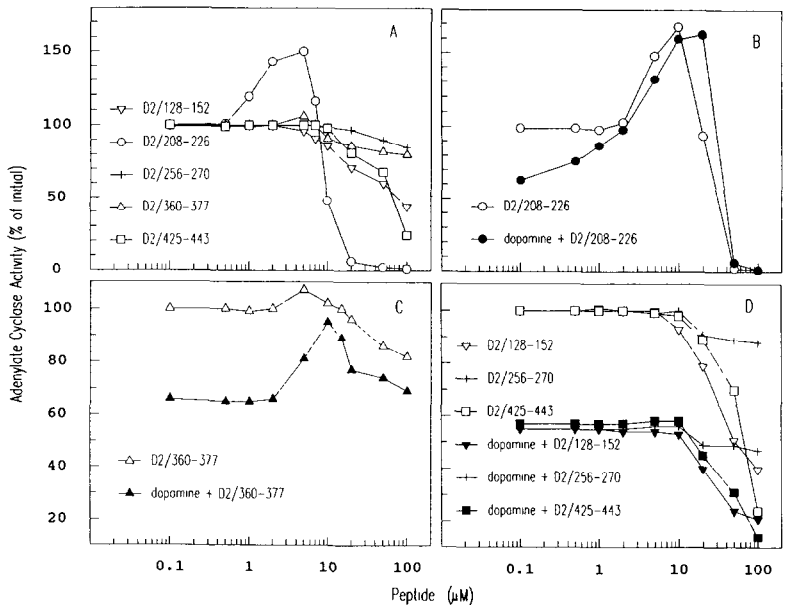

Fig. 2. (A) Effects of synthetic peptides on prostaglandin E_1 mediated adenylate cyclase activation in membranes from non-transfected 293 cells. (B–D) Effects of synthetic peptides on dopamine dependent adenylate cyclase inhibition in membranes of 293 cells expressing the dopamine $D_{2(long)}$ receptor. Data are presented as percentage of initial adenylate cyclase activity obtained with 1 μM PGE_1 in the absence of dopamine and peptides; maximal cAMP levels were 70 ± 10 pmol/assay for $D_{2(long)}R$ and 60 ± 10 pmol/assay for $D_{2(short)}R$. PGE_1 (open symbols) or PGE_1 and dopamine (filled symbols) were added to membranes preincubated with synthetic peptides.

(Fig. 2B) and D2/360–377 (Fig. 2C). The peptide D2/208–226 attenuates dopaminergic adenylate cyclase inhibition already at concentrations lower than 1 μM and overcomes the dopamine effect at 2 μM. Above 2 μM adenylate activity was increased (Fig. 2A). Effects on D_2R decoupling elicited by D2/360–377 require somewhat higher doses (2–10 μM). Other effects observed at even higher concentrations of all peptides might be caused by their direct interaction with a broad range of G proteins since these effects are of comparable size in membranes from transfected as well as non-transfected cells and appear independent on the presence of D_2R and hormone (compare Fig. 2A and D).

The potency of some receptor mimetic peptides to elicit effects in G proteins deserves special attention in view of the recent observations of Gilman and Bourne, who found that adenylate cyclase (type II or IV) activity will be activated by G protein $\beta\gamma$ subunits provided that G_s is also active [10,22]. Since in 293 membranes used in this study G_s-mediated preactivation of adenylate cyclase is necessary, stimulation of other G proteins which makes $\beta\gamma$ subunits available might be sufficient to stimulate or inhibit adenylate cyclase.

In contrast, peptides comprising loop i2, the middle part of loop i3 and the COOH-terminus failed to cause changes in dopaminergic adenylate cyclase inhibition at 0.1–10 μM (Fig. 2D).

3.3. *Effects of D2-peptides on GTPase activity in membranes*

The peptides D2/208–226 (Fig. 3A) and D2/360–377 (Fig. 3B) caused a significant increase of the GTPase activity in transfected 293 membranes. The up to 2-fold stimulation of GTPase activity was independent of the presence of dopamine suggesting a direct interaction of the peptides with G_{i2} or related G-proteins. A decrease of P_i formation caused by D2/208–226 was observed at concentrations beyond the range where adenylate cyclase inhibition was decoupled (Figs. 2B and 3A). Peptides D2/128–152, D2/256–270 and D2/425–443 showed no significant effect in the same concentration range (Fig. 2C). The fact, that only peptides affecting adenylate cyclase were also effective in GTPase activation, is in good agreement with our assessment of the adenylate cyclase inhibition studies.

4. DISCUSSION

To assess the topology of intracellular receptor sequences involved in determining G protein selectivity we focused on a comparison of differential responses to synthetic peptide fragments of two receptor prototypes both modulating adenylate cyclase activity: βAR functioning primarily as a stimulatory receptor and D_2R functioning predominantly as an inhibitory receptor.

Supplementing earlier investigations, our results clearly display which sites in βAR [3] or D_2R are potentially involved in G protein interaction and which sites can be excluded. Previously, we could not find effects elicited by peptides from loop i1 of βAR, except a very weak inhibition [1], which might be due to partial similarity to a motif composed of positively charged resi-

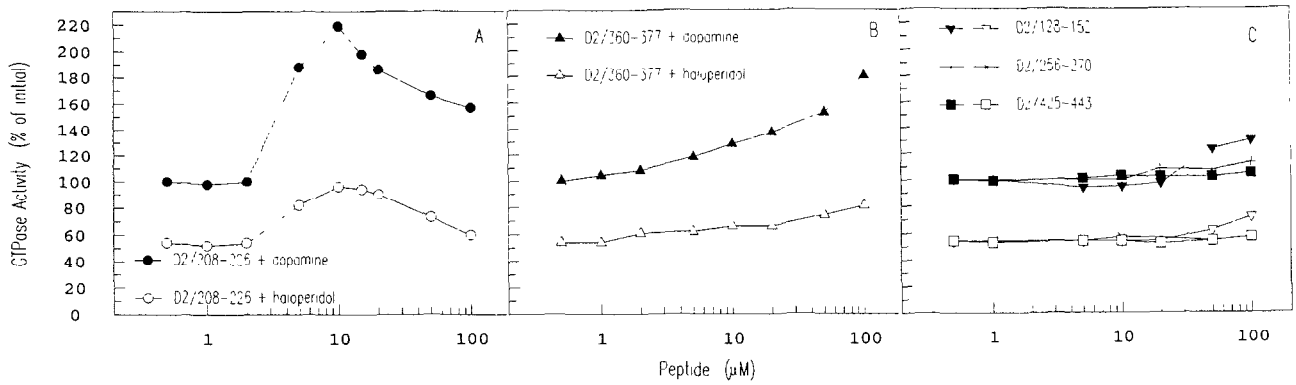

Fig. 3. Effects of synthetic peptides on GTPase activity in membranes from 293 cells expressing the dopamine $D_{2(long)}$ receptor Initial GTPase activity was measured at 100 μM dopamine without peptides. The maximal dopamine-stimulated $^{32}P_i$ levels obtained after 10 min incubation were 1.08 ± 0.15 pmol/assay for $D_{2(long)}$R and 1 14 ± 0.15 pmol/assay for $D_{2(short)}$R Membranes were incubated with 100 μM dopamine (filled symbols) or 10 μM haloperidol (open symbols).

dues interspaced by neutral residues [23]. Therefore, cryptic functions of loop i1 were not studied with peptides from D_2R. Peptides from loop i2 were effective only in the stimulatory branch. Effects caused by the N-terminal and C-terminal peptides of loop i3 indicate the essential character of these regions for signal transfer in both prototypes. Supplementary studies with a peptide representing part of the insert in loop i3 of $D_{2(long)}$)R, which has been hypothesized to encode part of the specifying message, were negative; these results imply that the insert does not engage in direct interactions with a binding site on a G protein. Accordingly, all results using either membranes from $D_{2(long)}$- or $D_{2(short)}$-transfected cells were identical. Small differences in receptor–G protein coupling between the short and long forms of D_2R were reported by Castro and Strange, presumably reflecting differences in structure of loop i3 [17]. Finally, peptides derived from loop i4 were effective only in case of βAR. Since the D_2R does not extend beyond loop i4, common receptor–G protein interaction sites are unlikely to be located in the far C-terminal region of G protein linked receptors.

Unlike the well-documented contributions from the 2nd, 3rd and 4th inner loops of the structurally related βAR to signal transduction in the stimulatory branch of adenylate cyclase control [1,3], we could identify and locate only two regions within the loop i3 that are involved in dopaminergic inhibition of adenylate cyclase activity in membranes. Therefore, it appears that a rather limited number of subsites are already sufficient to specify the interactions in the inhibitory branch. The present results do not finally settle the question of how many receptor sites are required to confer selectivity for signal transduction; however, as far as conserved membrane-attached sites of inner loops are involved, the present selection of peptides provides a complete coverage of all potential sites. No interaction sites have been reported for the non-conserved parts of loop i3, moreover, these regions can be removed by protcolysis or deletion mutagenesis without loss of regulatory function [9].

Analysis of the results from adenylate cyclase modulation and GTPase activation or inhibition allows some further specification of the presumed functional interactions of loop i3 regions. The effects of D2/208–226 below 2 μM apparently quench dopaminergic inhibition and thus appear to be specific for D_2R–G protein coupling; above 2 μM the peptide stimulates adenylate cyclase activity even in non-transfected membranes. The effects of D2/360–377 (Fig. 2C) below 10 μM are equally specific in attenuation of the dopaminergic inhibition of adenylate cyclase. It is tempting to speculate that the observed dose dependence of different G protein associated effects might also play a role in specifying the selective response. In the present case, interaction with the N-terminal region of loop i3 (D2/208–226) makes the response specific for dopaminergic adenylate cyclase inhibition, consequent interaction of a second receptor domain (C-terminal part of loop i3, represented by D2/360–377) will be confined to the same G protein molecule, whatever the preference of the synthetic peptide at higher concentrations may be [24].

G protein interactive sites were consistently found in all related G protein receptors like rhodopsin [5], β_2AR [6], M_4AChR [23] and α_2AR [25]. The fact that many of these sites share conserved sequences within related receptors and therefore must share conserved recognition sites on the G protein surface was explored for proposing one or a few consensus motifs for G protein interaction [23]. Consequently, in addition to G protein interactive peptides, such as venom peptide toxins [26,27] and neuroactive peptides [28], sequences of single transmembrane spanning receptors were found which are similarly interactive with G proteins [7]. The latest acquisition to these sequences was the interaction of β-amyloid precursor protein (APP) derived se-

quences or their mutations with G_o [8]. It is still a matter of speculation if the latter interactions are essentially mimetic or constitute a second class of receptor–G protein signaling, or serve the regulation of membrane traffic [8,29]. Unlike the at least two-sited interaction of conventional G protein receptors, a single site interaction of the latter is sufficient to elicit a G protein response.

In view of these considerations the present study can make a valuable contribution for the evaluation of the topology of conventional G protein receptor interaction sites, especially towards a discrimination of the stimulatory and inhibitory path of adenylate cyclase control. Our present results and related studies from this and other laboratories can be treated as special cases of a more general strategy to confer specificity and selectivity to receptor–G protein coupling [1,3,5,30,31]. To meet these requirements all receptors share distinct G protein binding domains which reflect structural similarities of heterotrimeric G protein subtypes; on the basis of related homologous sequences, single domains might be slightly altered to provide selectivity [9]. There is a need for multiple points of interaction; the organisation of these domains may vary between different classes of G protein-linked receptors. Both points are of special relevance for a selective interaction with a distinct member from a variety of G proteins. Examples for the general case were provided by rhodopsin–G_t [5] and βAR–G_s [3] interaction; in both cases effects of the corresponding receptor peptides were synergistic and supported a simultaneous multisite attack. In receptors which are functionally linked to attenuation of adenylate cyclase activity like D_2R two sites in loop i3 are sufficient for G protein selectivity [23,30–32].

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